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1. Cancer Research:
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   1987 Jul-Aug, 7(4B):781-789
   1997, Vol. 17, No. 3C, pp. 1973-1983

3. Gynecologic Oncology:
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   1992 Jun, 45(3):273-278
   1991, Vol. 42, No. 1, pp. 39-43
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4. Pathobiology:
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   1993, 61(2):67-76
   1992, 60(1):33-41

5. Jikeikai Med J:
   1989, 36(4):303-316
   1994, 41(4):407-415

6. Sapporo Med J:
   1988, 57(6):603-612
   1996, 65(5):433-444

7. Acta Obstetricia et Gynecologica Scandinavica, 1995, 74(5):330-335

8. Journal of Medicine, 1997, 28(3-4):175-190

9. Experimental and Molecular Pathology, 2000 Dec, 69(3):175-191

10. International Journal of Cancer, 15 Mar 2001, 91(6):778-782

11. Experimental Cell Research, 1992 Aug, 201(2):262-272

Thanks!

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Histog n sis and Culture f Human Ut rin Carcin sarc ma

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ABSTRACT

W set out to ascertain whether uterine carcinosarcoma r presents: (*a*) a "collision tumor," *i.e.*, a mixture of two histogenetically distinct malignant cell populations (endometrial carcinoma and sarcoma); (*b*) a "combination tumor" with both histological elements of common stem cell origin; or (*c*) a "composition tumor," *i.e.*, an endometrial carcinoma with reactive, atypical stroma. In *in vitro* cultures of human uterine carcinosarcoma, we could separate two distinct, different cell types and succeeded in establishing adenocarcinoma cell lines (HWUA-1 and HWUA-2) and sarcoma cell lines (HWUS-1, HWUS-1a, and HWUS-2). These cell lines grew well for over 10 months. HWUS-1a was hypertetraploid, HWUA-1 and HWUA-2 were pseudodiploid, and HWUS-1 and HWUS-2 were hyperdip id. These cell lines were transplanted into the subcutis of CAnLB/c nude mice and produced tumors. HWUA-1 and HWUA-2 cells produced poorly differentiated adenocarcinoma, HWUS-1 and HWUS-2 produced poorly differentiated sarcoma, and HWUS-1a produced well-differentiated leiomyosarcoma. These results support the combination tumor theory and reject the composition tumor theory as the cause of carcinosarcoma.

INTRODUCTION

Histogenesis of the uterine carcinosarcoma has been studied by many investigators, and various theories of origination have been proposed: from the Wolffian duct by Wilms (22); from embryonic cell rest of the Müllerian duct (6, 14, 20); from metaplasia of endometrial interstitial cell (15); from multipotent primitive mesenchymal cell of endometrial interstitial tissues (1, 11–13, 17), etc.

Meyer (10) mentioned possibilities of collision theory, combination theory, and composition theory for coexistence of carcinoma and sarcoma in carcinosarcoma. "Collision tumor" is a mixture of 2 histogenetically distinct malignant cell populations that have arisen in separated primary sites such as endometrium and stroma. "Combination tumor" is composed of both histological elements of common stem cell origin. Combination theory was supported by Norris *et al.* (11), Norris and Taylor (12), and Sternberg *et al.* (18). "Composition tumor" is an endometrial carcinoma with reactive, atypical stroma. The composition theory was supported by Ewing (3), Harvey and Hamilton (4), and Willis (21). Many other theories (5, 22) have since been reported, but no definite conclusion ha been given as yet.

We therefore attempted culture of human uterine carcinosarcoma in order to clarify its histogenesis and established the cell lines for adenocarcinoma cells and sarcoma cells, respectively; the cytological characteristics of each of the cell lines are reported here with discussion of the histogenesis of carcinosarcoma based on the findings.

MATERIALS AND METHODS

Materials. A 50-year-old woman underwent hysterectomy and bilateral salpingooophorectomy on June 22, 1979. The tumors existed separately at the lower part (Fig. 1*A*) and the upper part (Fig. 1*B*) of uterine corpus and bilateral ovaries (Fig. 1). The tumor tissues of the lower and the upper part of uterin corpus were placed into culture on June 22, 1979. The tumors were stained by hematoxylin and eosin, PTAH[1] (2), VG (19), and MA (5).

Tissue Culture Techniques and Culture Media. The culture materials were rinsed several times with culture medium and cut into fragments with razor blades. The fragments were washed several times with Hanks' solution containing dispase (500 picounits/ml) (Godo Syusei Co., Ltd., Tokyo, Japan) for 30 min at room temperature, aspirated by plastic injector equipped with fine needles (from 17 to 21 gauge), and centrifuged at 1600 rpm for 10 min. The sediments were resuspended in the culture medium, placed in plastic dishes (Falcon Plastics, Oxnard, Calif.), and finally incubated at 37° in a humidified atmosphere containing 5% CO_2:3% O_2 in air. The medium was 85% Ham's F-10 (Grand Island Biological Co., Grand Island, N. Y.):10% newborn calf serum:2.5% calf serum. 2.5% horse serum at pH 7.4, supplemented with 50 units penicillin and 50 μg streptomycin per ml. The cells were transferred at 1:2 or 1:4 dilution, using 0.25% trypsin solution (Difco Laboratories, Detroit, Mich.) in Hanks' balanced salt solution (Flow Laboratories, McLean, Va.).

Growth Curve, Doubling Time, and Plating Efficiency Assays. For growth curve studies, about 1×10^5 single-suspension cells per ml were plated onto 3.5-cm plastic dishes (Falcon). They were incubated for about 7 days, and the average number of cells was determined every day by counting cells in 3 dishes. The medium was changed every 2 days. The population doubling time was determined by the growth curve (19). For studies of plating efficiency, 1×10^2 single-suspension cells were incubated in 5 plastic dishes (6.5 cm in diameter; Falcon) for 10 days. The cells were stained with Giemsa solution (Kokusan Chemical Works, Ltd., Tokyo, Japan). Plating efficiency was determined by the ratio of the number of colonies to the total number of inoculated cells.

Light Microscopic Observation. The cultured cells were fixed with 95% ethanol solution, stained with Papanicolaou stain, and also fixed with 10% formalin solution and stained with PTAH, VG, and MA.

Chromosomal Analysis. The cells were treated with 1×10^{-7} M Colcemid (Ciba, Ltd., Basel, Switzerland) for 8 hr at

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[1] The abbreviations used are: PTAH, phosphotungstic acid:hematoxylin; VG, Van Gieson; MA, Heidenhain Mallory azan.

37°, placed in a 0.2% KCl solution for 15 min, and then fixed with alcohol:acetic acid (3:1) solution. More than 100 metaphase plates were examined after being stained with buffered Giemsa solution. Their kary types were strictly analyzed in accordance with the recommendati ns of the Paris Conf r-ence.

Heter transplantation. Approximat ly 1 × 10^6 suspended cells were transplanted s.c. into BALB/c nude mice (8 weeks ld; Nisseiken Co., Ltd., Tachikawa, Japan) (8). The tumors were examined histologically 1 month after transplantation.

RESULTS

Pathology of Culture Materials. The tumor of the lower part (Fig. 1*A*) of the uterus was composed of spindle- or fibrous-shaped cells and showed neoplastic and pleomorphic features. The cytoplasm was stained deep blue by PTAH, yellow by VG, and red by MA, and it contained fibrous materials without cross-striation. These histological appearances were interpr ted as leiomyosarcoma. The carcinomatous elements coxisting in the same tumor were poorly differentiated adenocarcinoma. Disloc tion did not exist between sarcomatous and carcinomatous elements. The tumor was finally diagnosed as carcinosarcoma (Fig. 2). The tumors of the upper part (Fig. 1*B*) of th uterus and of bilateral ovaries were interpreted as poorly differentiated adenocarcinoma.

M rphology of Cultured Cells. The epithelial cells (HWUA-1) and the nonepithelial cells (HWUS-1) were separated completely from initial culture (Fig. 3) of the lower tumor by a single-cell plating method using feeder layer techniques in Rose's chamber (7) on August 2, 1979. The HWUA-1 cells appeared epithelial, showing a pavement arrangement and spindle or polygonal shape (Fig. 4), and grew in multilayers. Th HWUS-1 cells were spindle or elongated fibrous in shape and grew as multilayers without contact inhibition (Fig. 5). The stress fiber-like structures existed in the long fibrous cells and stained deep blue by PTAH, yellow by VG, and red by MA. The HWUS-1a cell line was established from the HWUS-1 cells by using cloning techniques (7) on October 8, 1979. The HWUS-1a cells showed more neoplastic features such as coarse granular chromatin, prominent nucleoli, and irregular nuclear membrane than did HWUS-1 cells. The HWUA-2 and the HWUS-2 cell lines derived from the upper tumor were established with cloning methods (7) on August 2, 1979. The HWUA-2 and HWUS-2 cells were similar in features to the HWUA-1 and HWUS-1 cells.

Growth Characteristics. The population-doubling time and plating efficiency of each cell line are shown in Table 1. The growth rate was then accelerated with subcultivation. Each cell lin grew well without interruption, was passed more than 50 times within 10 months, and continues to be stable growth.

Chromosomal Analysis. Chromosomal analysis was done at passages 5 and 20 of each cell line (Table 2). The chromosomal number of HWUA-1 and HWUA-2 was widely distributed, and the modal number was 46. The marker chromosome was not seen. The chromosomal number of HWUS-1 and HWUS-2 was also widely distributed, and the modal number was 47. Howev r, that of HWUS-1a was distributed at th narrow range from 88 to 108, and the m dal number was 95. Less than 3% of HWUS-1 and HWUS-2 cells had abn rmal larg subtelocentric chromosomes, and mor than 75% of HWUS-1a cells had abnormal large subtelocentric marker chromosomes and minute chromosomes (Fig. 6).

Table 1
Growth characteristics of HWUA-1, HWUA-2, HWUS-1, HWUS-1a, and HWUS-2 cell lines

Cell line	Total PN[a]	Culture periods (mos.)	PN	DT (hr)	PE (%)
HWUA-1	85	13	10	41	65
			30	33	68
			50	31	66
HWUA-2	68	13	10	40	66
			30	32	68
			50	31	69
HWUS-1	63	13	10	46	42
			30	34	40
			50	31	40
HWUS-1a	58	10	5	30	35
			30	26	34
			50	24	36
HWUS-2	67	13	10	38	43
			30	31	44
			50	29	45

[a] PN, number of passages; DT, doubling time; PE, plating efficiency.

Heterotransplantation. One month after transplantation, these cells produced tumors in the subcutis of BALB/c nude mice. The tumors produced by HWUA-1 and HWUA-2 cells were histologically interpreted as poorly differentiated adenocarcinoma (Fig. 7). The tumors produced by HWUS-1 and HWUS-2 cells indicated poorly differentiated sarcoma (Fig. 8). That of HWUS-1a indicated well-differentiated leiomyosarcoma. The cytoplasm of giant fibrous cells in HWUS-1a tumor contained myofilaments which were stained deep blue by PTAH, yellow by VG, and red by MA.

DISCUSSION

A carcinosarcoma is a solitary tumor (12) in which constituent carcinoma and sarcoma exist as a diffuse mixture with no dislocation as the boundary of the 2 kinds of tumor; this is originally the morphological characteristic of carcinosarcoma. Collision tumors are not true carcinosarcoma, but the 2 lesions have been confused in the past (18, 21). The collision tumor is characterized as having dislocation between carcinoma and sarcoma. Dislocation does not exist between the 2 elements in the combination tumor, because the 2 elements (carcinoma and sarcoma) originate from a common stem cell (12, 13). Dislocation also does not exist in the composition tumor (12). Therefore, we incline to the combination tumor theory or composition tumor theory, histologically, and exclude collision tumor theory from the histogenesis of carcinosarcoma. Histogenesis, however, is still obscure with histological observations only.

It would enable us to clarify the histogenesis of carcinosarcoma if we can decide definitely by analysis whether (*a*) 2 histogenetically distinct malignant cell populations (endometrial carcinoma and sarcoma) exist or (*b*) endometrial carcinoma exists only in carcinosarcoma. Thus, we attempted culture of ut rine carcinosarcoma (coexistence of poorly differentiated adenocarcinoma and leiomyosarcoma), identified the cultured cells not only by morphological examinations but also by chromosomal analysis and heterotransplantation, and established

Table 2

Distribution of chromosome number of HWUA-1, HWUA-2, HWUS-1, HWUS-2, and HWUS-1a cells

No. of chromosomes	HWUA-1		HWUA-2		HWUS-1		HWUS-2		HWUS-1a	
	Passage 5	Passage 20	Passage 5	Passage 20	Passage 5	Passage 20	Passage 5	Passage 20	Passage 5	Passage 20
41			1		2	1	2	2		
42	2	2	1	1	1	2	2	1		
43	3	3	2	2	3	3	4	2		
44	3	7	6	4		1	5	3		
45	10	10	11	12	4	3	8	4		
46	16	18	20	20	14	15	14	17		
47	9	8	10	12	20	19	16	20		
48	7	7	5	8	15	15	14	14		
49	4	4	4	5	9	10	6	8		
50	5	5	3	1	13	12	3	2		
51	2	1			2	1	1	1		
52			3	2	1	1	1	1		
53		1								
54			2	1	1	1				
55						1				
62		1					1			
63				1						
64	1		1	1			1			
65				1			1			
66		1	1	1						
67	1				1	1	1	1		
68	2	2	3	3			2			
69	3	3	4	4	2	2	1	2		
70	2	3	2	3			1	3		
71	1	1	1							
72	2	1								
73			1	1						
74		1	1				1	1		
77							1			
80								1		
81	1	1					2			
82	1			1				1		
83			1							
87				1			1			
88	1	1	2	1				1	1	2
89	1						1		2	1
90	1	1	1		3	1	1		2	2
91	3	2	2	2	2	1		1	5	3
92	3	5	5	7	2	1	1	2	4	6
93	3	3	2	2	1	1	2	1	8	7
94	2	2	2	2	1	2	1	3	12	9
95			1			3	3	4	15	19
96	1	2						1	13	14
97									10	9
98									8	7
99									7	6
100									4	5
101									2	2
102									2	1
103									1	1
104										
106									1	
107									1	3
108									3	2
More than 110	3	3	2	1	3	3	2	3	2	1

3 kinds of cell line. HWUA-1 and HWUA-2 are nearly the same cells and are cultured for about 13 months after primary culture. They have the morphological characteristics of the epithelial malignant cell. Their chromosomes show a wide distribution in aneuploidy, and the cells can be heterotransplanted to nude mice to form adenocarcinoma on them. These characteristics enable us to identify the lines to be those from the adenocarcinoma. HWUS-1 and HWUS-2 are identified as sarcoma cell lines by morphological and chromosomal analysis and by heterotransplantation. HWUS-1a is a line of fibrous cells with myofilaments in the cytoplasm and is capable of forming leiomyosarcoma on nude mice; this enables us to identify it as a leiomyosarcoma cell line.

Based on our data obtained by culture of carcinosarcoma, it was found that carcinosarcoma is a combination tumor composed of 2 kinds of cells, carcinoma cells and sarcoma cells, and is not a composition tumor composed of endometrial carcinoma only with reactive, atypical stroma. Rubin (16) also rejected the composition theory as the cause of this tumor after tissue culture of mixed mesodermal tumor in which poorly differentiated carcinoma and rhabdomyosarcoma coexisted as a mixture, based on his findings that 2 kinds of cells, epithelial malignant cells (carcinoma) and nonepithelial malignant cells (sarcoma), proliferated from the tissue segments. However, the cultured cells died after 2 weeks of culture, making long-term cultivation unsuccessful.

Since no report has been found on the establishment of a long-term cultivation line of human uterine carcinosarcoma, th

present report is expected to shed light on studies on the effect of anticancer agents, on the mechanism of myosin synthesis, etc.

REFERENCES

1. Chuang, J. T., Van Velden, D. J. J., and Graham, J. B. Carcinosarcoma and mixed mesodermal tumor of uterine corpus. Review of 49 cases. Obstet. Gynecol., *35:* 769–780, 1970.
2. Debell, C. Some new methods for studying intestinal amoebae and other protozoa. Parasitology, *34:* 101–110, 1942.
3. Ewing, J. Clinical types of sarcoma. *In:* J. Ewing (ed.), Neoplastic Diseases, pp. 286–287. Philadelphia: W. B. Saunders Co., 1934.
4. Harvey, W. F., and Hamilton, T. D. Carcinosarcoma, a study of the microscopic anatomy and meaning of peculiar cancer. Edinb. Med. J., *42:* 337–378, 1935.
5. Heidenhain, M. Über die Mallorysche Bindegewebsfärbung mit Karium und Azokarmin als Vor Färben. Z. Wiss. Mikrosk. Mikrosk. Tech., *32:* 361–370, 1915.
6. Hill, R. P., and Miller, F. M., Jr. Combined mesenchymal sarcoma and carcinoma (carcinosarcoma) of the uterus. Cancer (Phila.), *4:* 803–816, 1951.
7. Ishikawa, H., Shino, M., Arimura, A., and Rennels, E. G. Functional clones of pituitary cells derived from Rathke's pouch epithelium of fetal rats. Endocrinology, *100:* 1227–1230, 1978.
8. Ishiwata, I., Nozawa, S., Nagai, S., Kurihara, S., Mikata, A., and Okumura, H. Establishment of a human leiomyosarcoma cell line. Cancer Res., *37:* 658–664, 1977.
9. Ishiwata, I., Udagawa, Y., Okumura, H., and Nozawa, S. Effects of progesterone on human endometrial carcinoma cells *in vivo* and *in vitro*. J. Natl. Cancer Inst., *60:* 947–954, 1978.
10. Meyer, R. Mischgeschwülste des Uterus; die Komplizierten Tumoren. *In:* F. Henke and . Lubarsch (eds.), HandBuch der Speziellen Pathologischen Anatomie und Histologie, pp. 412–415. Berlin: Julius Springer, 1930.
11. Norris, H. J., Roth, E., and Taylor, H. B. Mesenchymal tumors of the uterus. II. A clinical and pathologic study of 31 mixed mesodermal tumors. Obstet. Gynecol., *28:* 57–63, 1966.
12. Norris, H. J., and Taylor, H. B. Mesenchymal tumors of the uterus. III. A clinical and pathologic study of 31 carcinosarcomas. Cancer (Phila.), *19:* 1459–1465, 1966.
13. Novak, E., and Anderson, D. F. Sarcoma of uterus. Am. J. Obstet. Gynecol., *34:* 740–781, 1937.
14. Ober, W. B. Uterine sarcomas—histogenesis and taxonomy. Ann. N. Y. Acad. Sci., *75:* 568–585, 1959.
15. Pfannenstiel, J. Das traubige Sarcom der Cervix Uteri. Virchows Arch. Pathol. Anat. Physiol. Klin. Med., *127:* 305–337, 1892.
16. Rubin, A. The histogenesis of carcinosarcoma (mixed mesodermal tumor) of the uterus as revealed of tissue culture studies. Am. J. Obstet. Gynecol., *77:* 269–274, 1959.
17. Silverberg, S. G. Leiomyosarcoma of the uterus. A clinicopathologic study. Obstet. Gynecol., *38:* 613–623, 1971.
18. Sternberg, W. H., Clark, W. H., and Smith, R. C. Malignant mixed Müllerian tumor (mixed mesodermal tumor of the uterus). Cancer (Phila.), *7:* 707–724, 1954.
19. Van-Gieson, J. Laboratory results on technical method for the nervous system. N. Y. Med. J., *50:* 57–65, 1889.
20. Vellios, F., Stander, R. W., and Huber, C. P. Carcinosarcoma (malignant mixed mesodermal tumor) of the uterus. Am. J. Clin. Pathol., *39:* 495–505, 1963.
21. Willis, R. A. Structure and growth of tumours. *In:* R. A. Willis (ed.), Pathology of Tumours, pp. 139–141. London: Butterworth Co., 1953.
22. Wilms, M. Die Mischgeschwülste. Heft II; Die Mischgeschwülste der Vagina and Cervix Uteri (Traubige Sarcome). Anhang: Mischgeschwülste der Blase und des Vas Deferens, pp. 153–156. Leipzig, Germany: A. Georgi, 1900.

Fig. 1. Gross appearances of operation specimens. *A*, the lower tumor (carcinosarcoma); *B*, the upper tumor (adenocarcinoma).

Fig. 2. The histology of the original tumor of the lower part (Fig. 1*A*) of uterus showing carcinosarcoma. H & E, × 100. *A-1*, sarcoma. H & E, × 400. *A-2*, adenocarcinoma. H & E, × 400.

Fig. 3. Primary cultured cells of the lower tumor formed an epithelial colony (*A*) surrounded by fibroblastic cells (*S*). Phase-contrast microscopy, × 150.

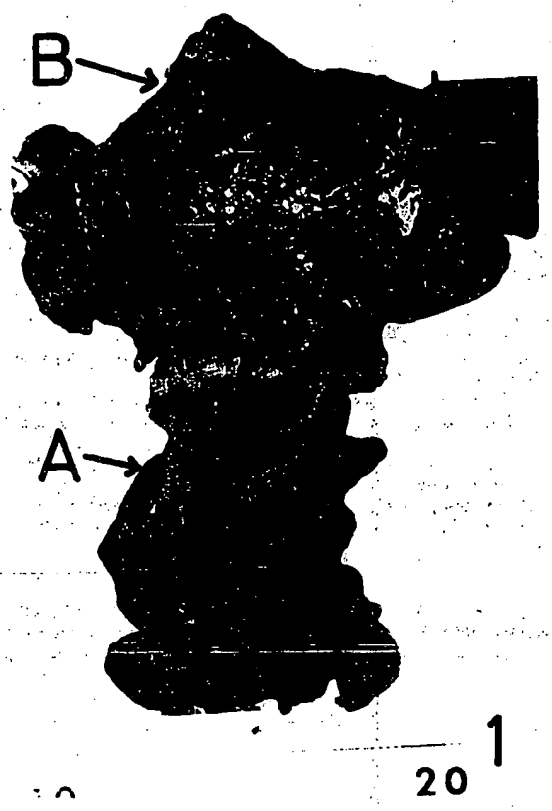
Fig. 4. HWUA-1 cells (passage 20) were polygonal shaped, but multinucleated giant cells also appeared and revealed anaplastic and pleomorphic features. Cytoplasms were observed to have vacuoli (periodic acid-Schiff positive). Papanicolaou, × 100.

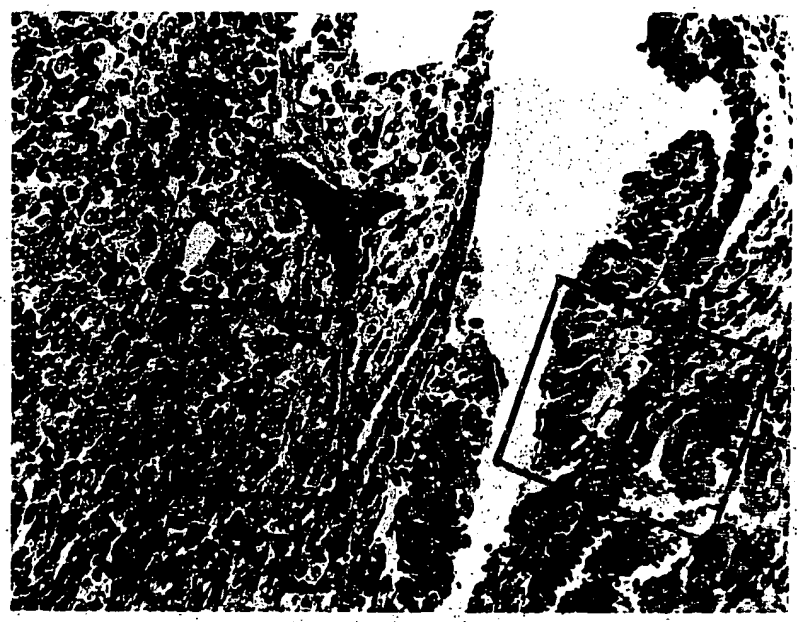
Fig. 5. HWUS-1 cells (passage 20) were fibrous in shape, revealed anaplastic and pleomorphic features, and grew multilayered. Papanicolaou, × 80.

Fig. 6. Metaphase chromosomes of passage 5 HWUS-1a cells show hyperploidy and large subtelocentric marker chromosome (*M*) and minute chromosome (*m*). Giemsa, × 1,500.

Fig. 7. The histology of tumor produced by passage 20 HWUA-2 cells was interpreted as poorly differentiated adenocarcinoma. H & E, × 150.

Fig. 8. The histology of tumor produced by passage 20 HWUS-2 cells was interpreted as poorly differentiated sarcoma. H & E, × 150.


B
A
20
1

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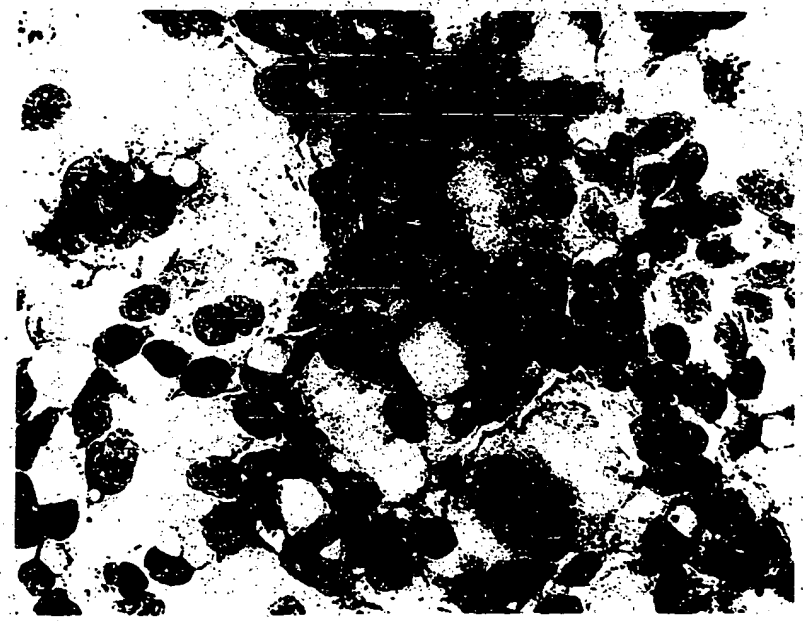
S



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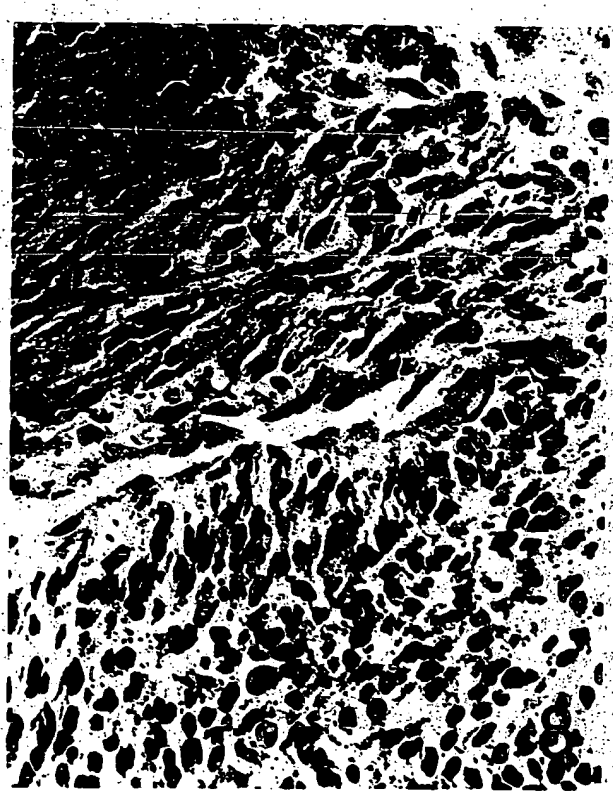

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